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ORIGINAL PAPER

Control of *Fusarium* wilt of tomato caused by *Fusarium oxysporum* f. sp. *lycopersici* using leaf extract of *Piper betle* L.: a preliminary study

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Abstract The main objective of this study was to evaluate the effectiveness of crude chloroform extract of *Piper betle* L. (PbC) in controlling *Fusarium* wilt of tomato (*Lycopersicon esculentum*) caused by *Fusarium oxysporum* f. sp. *lycopersici*. It was observed that 1% (w/w) amendment of the PbC in soil was more efficient in reducing the *Fusarium* population in soil than carbendazim and the combined amendment of carbendazim and PbC. *Fusarium* wilt control studies were carried out in a greenhouse. Variation in different parameters like shoot growth, root growth and mean fresh weights of tomato seedlings in all the treatments were recorded. Accumulation of total phenolics was also studied from the root tissues of tomato. Higher accumulation of total phenolics was observed in the *Fusarium*-infested plants as compared to that of healthy control and PbC-treated plants. Moreover, it was observed that the extract could reduce the symptoms and disease development. Electron microscopy studies were also done to observe the *Fusarium* infestation in the vascular bundles and to show the accumulation of total phenolics in the vacuoles of root tissue.

Keywords *Fusarium* wilt · *Piper betle* L. · Tomato

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Introduction

Fusarium species causes a huge range of diseases on an extraordinary range of host plants. The fungus can be soilborne, airborne or carried in plant residue and can be recovered from any part of the plant from the deepest root to the highest flower (Booth 1971; Summeral et al. 2003). *Fusarium* wilt of tomato (*Lycopersicon esculentum*) caused by *Fusarium oxysporum* f. sp. *lycopersici* is a disease that causes serious economic loss (Agrios 2005). The fungus causes vascular wilts by infecting plants through the roots and growing internally through the cortex to the stele (Bowers and Locke 2000). Control of wilt diseases depends mainly on fungicides (Minton 1986; DeVay et al. 1988). Several fungicides have been used for control of different plant pathogens including fusaria (Liggit et al. 1997; Diehl and Fehrmann 1999) and the number of effective fungicides with negligible effect on the environment is rare. Fungicides are expensive, can cause environmental pollution and may cause the selection of pathogen resistance (Lumsden and Locke 1989). Alternative methods of controlling the disease have been studied with emphasis on novel compounds derived from plant sources (Garibaldi et al. 1990; Alabouvette 1999). Plant extracts and plant essential oils have been reported to be effective antimicrobials against food and grain storage fungi, foliar pathogens and soilborne pathogens (Bowers and Locke 2000). Many plants and their products have been reported to possess pest control properties. These are good alternatives to chemical pesticides, as they are readily biodegradable in nature. In our previous study, we observed that chloroform extract of *Piper betle* L. (PbC) was found to be effective in controlling phytopathogenic fungi of economic importance in vitro (Singha et al. 2010). So, an attempt was made to study the efficacy of the *Piper* extract to control

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the *Fusarium* wilt of tomato in vivo. In this study, we report the effectiveness of crude chloroform extract of *P. betle* L. (PbC) in reducing populations of *F. oxysporum* in soil and controlling wilt development in tomato plants.

Materials and methods

Inoculum preparation

Fusarium oxysporum F1 (Acc. No. HM802271), F2 (Acc. No. HM802272) and F5 (Acc. HQ332534) were used in this study. For inoculum preparation, the fungus was cultured in potato dextrose broth (PDB; Himedia, Mumbai) enriched with yeast extract (Himedia, Mumbai) at 10 g l^{-1} in shake culture (150 rev min^{-1}), for 10 days at 28 ± 2°C. The broth culture was filtered through 4 layers of muslin cloth, centrifuged at 6,000*g* for 10 min and washed with sterile water. The test inoculum was adjusted to 2.1 × 10^6 spores per ml using a haemocytometer. Inoculum consisted mainly of microconidia.

Plant material

Leaves of *P. betle* L. were collected and shade dried. It was grounded into fine powder and solvent extracted using petroleum ether for 48 h. It was then filtered using filter paper no. 1 (Whatman, Mumbai) and the residue thus obtained was dried completely. It was again extracted using chloroform (Merck, Mumbai) for 48 h and filtered. The filtrate was dried in a rotary evaporator (Heidolph, Germany). The dried crude extract was diluted in appropriate concentrations of dimethylsulfoxide (Himedia, Mumbai). Qualitative analysis of the chloroform extract of *P. betle* L. was carried out using standard methods. Seeds of the *Fusarium* wilt susceptible tomato plant (Pusa Ruby) were surface disinfected with 1% sodium hypochlorite (Himedia, Mumbai) for 2 min and rinsed three times with sterile distilled water prior to sowing.

Soil infestation and treatment

Potting mixtures (pH 6.8) which is amended with KNO_3 (0.3 g l^{-1}), KH_2PO_4 (0.18 g l^{-1}) and NH_4NO_3 (0.3 g l^{-1}) was used. Sterile potting mixture was infested with spores of *Fusarium oxysporum*. It was incubated at 28 ± 2°C in sterile plastic bags for 5–7 days or till the fungal population reached 10^6 spores per gram of the soil. Uninfested soil was used as the negative control. For control of *Fusarium* wilt, the fungus-infested soil was treated with different concentrations of chloroform extract of *P. betle* L. (PbC) and carbendazim (BASF, Mumbai) under standard conditions. The experiment was carried out in a greenhouse.

Control of Fusarium wilt

For control of *Fusarium* wilt in the greenhouse, six-week-old tomato seedlings were planted in plastic bags (12 cm diameter; 1 l capacity) containing soil treatments. The treatments included: (a) healthy control (no fungus); (b) fungal infested control; (c) infested soil amended with DMSO; (d) infested soil amended with 0.1, 0.5, 1, 2 and 5% (w/w) PbC; (e) infested soil amended with 0.1, 0.5, 1, 2, and 5% (w/w) carbendazim and (f) infested soil amended with 0.1, 0.5, 1, 2 and 5% (w/w) combined PbC and carbendazim (1:1). Three different *Fusarium* isolates: highly virulent F1, virulent F2 and avirulent F5 were also included in the study to assess the activity of the plant extract on the *Fusarium* soil population and disease incidence. Soil samples of respective treatments were collected and the populations of the fungus were assessed in vitro using the soil dilution method (Aneja 2005). The soil dilutions were plated in pentachloronitrobenzene (PCNB; Sigma–Aldrich, Germany) agar medium (Nash and Snyder 1962), incubated at 28 ± 2°C for 3–5 days and appearance of *Fusarium* colonies was noted. Variation in shoot growth, root growth, fresh weight of seedlings and variation in symptom developments were observed in all the treatments for a period of 28 days after planting. The disease severity were recorded on a 0–3 visual scale, in which 0 = no symptoms; 1 = light yellowing of leaves; 2 = moderate or severe yellowing of leaves with or without wilting, stunting, severe rot on taproot and secondary roots, crown rot with or without hypocotyls rot and vascular discolouration in the stem; and 3 = dead seedlings (Vakalounakis and Fragkiadakis 1999). Disease incidence percentage was determined according to Song et al. (2004). The experiments were carried out in a greenhouse (22–28°C, 70–80% relative humidity and 12 h photoperiod). Each treatment consisted of 25 replicates and was repeated twice.

Determination of accumulation of phenolics in root tissue

The total phenolics content in tomato roots was determined using the Folin-Ciocalteu method (Policegoudra et al. 2007) with slight modifications. Briefly 100 µl of the methanolic root extracts of all treatments were mixed with 2 ml of 2% aqueous sodium carbonate solution (Himedia, Mumbai). After 5 min 100 µl of 50% Folin-Ciocalteu phenol reagent (SRL, Mumbai) was added to the mixture. Incubation was done at room temperature for 30 min and the absorbance was recorded at 750 nm against a blank using a spectrophotometer (Thermo Spectronic UV1, USA). Calibration curve was prepared using gallic acid (SRL, Mumbai) as the standard reference phenolic compound and thus total phenolics content was calculated. All

determinations were carried out in triplicates. The total content of phenolics in the root extract was calculated as μg g^{-1} fresh weight (FW) root tissue.

Electron microscopy analysis of tomato root tissue

Scanning electron microscopy (SEM) of root tissue was done using a JEOL JSM 6360 SEM operating at 20 kV. Transmission electron microscopy (TEM) was done using a JEOL JSM 100-CX TEM operating at 60 kV. These studies were carried out at Sophisticated Analytical Instrument Centre (SAIF), NEHU, Shillong, India.

Statistical analysis

Statistical calculations were carried out using one way analysis of variance (ANOVA) and the significances of the differences between means were calculated using Duncan's multiple range test under a significance level of $P < 0.05$.

Results

Qualitative phytochemical test

PbC showed the presence of saponins, phlobatanins, flavonoids, phytosterols, phenols, tannins and terpenoids.

Fusarium soil population assay

There was no *Fusarium* colony in the uninfested control (healthy) while in the *Fusarium*-infested control and DMSO control. There was almost the same number of *Fusarium* colonies. It was observed that PbC was more efficient than carbendazim and combined activity of carbendazim and PbC in reduction of the fungal population in all the treatments (Fig. 1). 1% PbC amendment in soil could successfully inhibit the *Fusarium* soil population. It was observed there was no significant difference in the inhibition of *Fusarium* population on the three different isolates: highly virulent *Fusarium* F1, virulent *Fusarium* F2 and avirulent *Fusarium* F5 by PbC (Table 1).

Effect of soil amendment with PbC on Fusarium

The growth of plants was stunted in fungus-infested soil as compared to the healthy normal control. Plants grown in *Fusarium*-infested soil amended with 1% (w/w) PbC attained greater shoot height (Fig. 2a) as compared to the diseased plants. It may be compared with that of the healthy control after 21 days of planting. The increase in the shoot height was statistically significant ($P < 0.05$) in all treatments on all days of observation. Seedlings planted

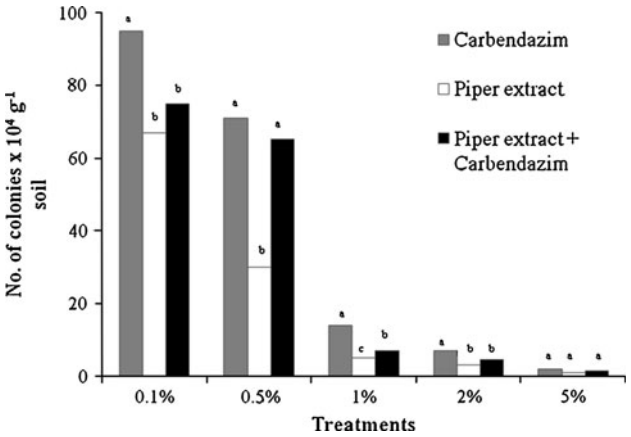

Fig. 1 *Fusarium* soil population assay in treatment with different concentrations of chloroform extract of *P. betle* L., carbendazim and combined treatment of chloroform extract of *P. betle* L. and carbendazim. Values with *different letters* are significantly different at $P < 0.05$

in *Fusarium*-infested soil without PbC amendment had decreased root growth. The root growth of seedlings planted in *Fusarium*-infested soil amended with 1% (w/w) PbC attained comparable growth with that of the healthy control after 28 days of planting. The results were statistically significant ($P < 0.05$) on all days of observation (Fig. 2b). Plants from *Fusarium*-infested soil amended with 2% (w/w) PbC attained comparable fresh weights with that of healthy control than plants from *Fusarium*-infested soil or the PbC treatment at 0.5% (w/w) PbC. The results were statistically significant ($P < 0.05$) (Fig. 2c). The PbC amendment in *Fusarium*-infested soil decreased the wilting symptoms and disease developments. The suppression of disease and symptom development for all treatments was significant on all days of observation. 1% (w/w) PbC amendment in soil gradually reduced the *Fusarium* population in soil. Only a negligible 10% disease incidence was observed after 28 days as compared to 65% disease incidence at 15 days of treatment (Fig. 2d). The results were statistically significant ($P < 0.05$) on all days of observation. It was also observed that delayed amendment of PbC in soil after 15 days of *Fusarium* F1 infestation could reduce the disease incidence form 88 to 44% only.

Determination of accumulation phenolics in root tissue

The fungus-infested seedlings had an increased content of total phenolics in their root tissues. The content increased to a maximum at 21 days in all treatments and then got gradually reduced except the fungal-infested seedlings. It was observed that there was a decline in the accumulation of total phenolics in the healthy control and the PbC amendments after 21 days of observation. But the *Fusarium-infested* treatments showed more accumulation of total

Table 1 Activity of chloroform extract of *Piper betle* L. on the population density and disease incidence of different *Fusarium oxysporum* isolates after 28 days of observation

Fusarium isolates	Population density (10^4 g^{-1} soil)		Disease incidence	
	Control	1% PbC amended soil	Control	1% PbC amended soil
F1 (extremely virulent)	107 ± 3.0a	4.6 ± 2.1a	88.8 ± 2.9a	11 ± 0a
F2 (virulent)	108 ± 4.6a	3 ± 1.8a	36.0 ± 8.0b	4.4 ± 2.7b
F5 (avirulent)	101 ± 2.0a	7 ± 2.3a	7.2 ± 3.4c	0 ± 0c

'*PbC*' chloroform extract of *Piper betle* L.; Values showed by different letters for each line is significantly different at $P < 0.05$. Values are given as mean values ± standard deviation

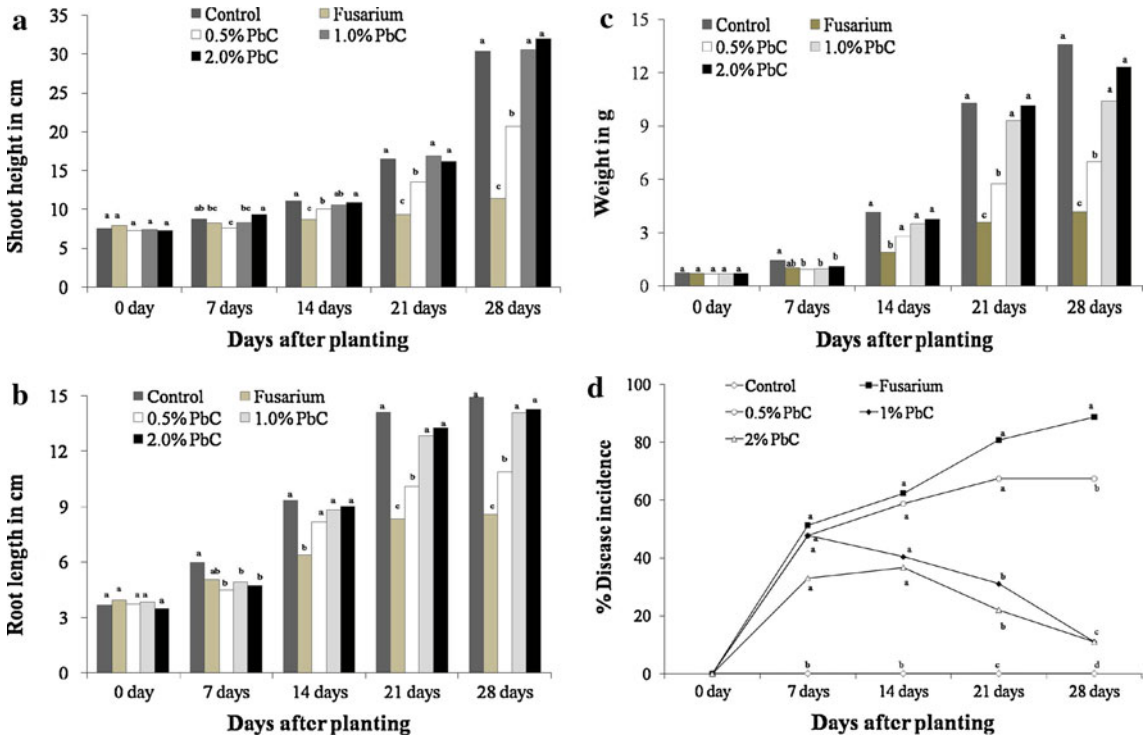

Fig. 2 Effect of tomato plants grown in soil amended with chloroform extract of *Piper betle* L. for control of *Fusarium* wilt. **a** variation in shoot growth; **b** variation in root growth; **c** variation in weight; **d** variation in disease incidence of tomato seedlings. Values showed by *different letters* for each line is significantly different at $P < 0.05$

phenolics even after 21 days of treatment, and reached the maximum at 28 days of observation (Fig. 3). The results were statistically significant ($P < 0.05$).

Electron microscopy analysis of tomato root tissue

It was evident from the scanning electron microscopic observations that *Fusarium* blocked the vascular bundles giving much insight into the fungal infestation in the *Fusarium-infested* tomato plants (Fig. 4b), whereas the healthy (Fig. 4a) and the PbC-amended seedlings showed clear vascular bundles (Fig. 4c). From the studies by transmission electron microscopy, a higher level of accumulation of total phenolics was observed in the vacuole of the *Fusarium-infested* tomato root tissue (Fig. 5b). The healthy control (Fig. 5a) and the 1% (w/w) PbC amendment had a normal accumulation of total phenolics. Also the healthy control was observed to have a cell wall with good lignifications (Fig. 5c) when compared with the cell wall of the *Fusarium-infested* root tissue (Fig. 5d).

Discussion

From our investigation, it was observed that there was a reduction in symptom development and as well as in *Fusarium* population after 28 days of observation in the tomato plants grown in soil treated with 1% (w/w) chloroform extract of *P. betle* L. (PbC). There was a significant increase in the shoot growth, root growth and mean fresh

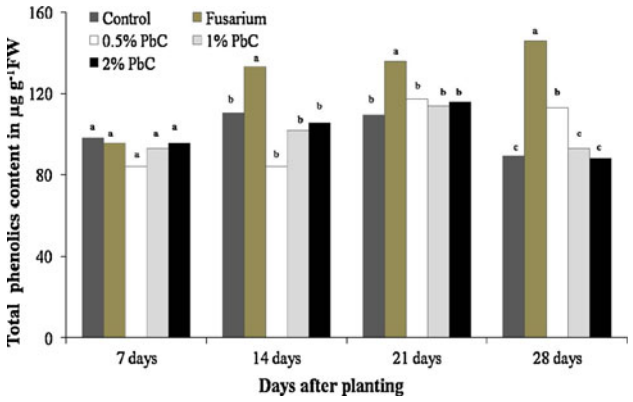

Fig. 3 Variation in accumulation of total phenolics in root tissue of tomato seedlings grown in soil with different amendments of chloroform extract of *Piper betle* L. Values showed by *different letters* for each line are significantly different at $P < 0.05$

weight of tomato plants as compared with the fungal control. This increase in all these parameters can be well compared with that of the healthy control on all days under observation. In the case of plants grown in soil amended with 2% (w/w) PbC, it was observed to be better than that of healthy control. It is a common phenomenon that *Fusarium* wilt generally attacks tomato plants during the growth period of 60 days, when flowering starts. The pathogen enters through the roots of the plant and proliferates in the vascular tissues leading to breakdown of the water economy of the infected plants (Agrios 2005). Singha et al. (2010) observed that the chloroform extract of *P. betle* L. was thermally stable at room temperature even up to 120 days of storage and more, with similar antifungal properties as the fresh extract. It was also observed that chloroform extract of *P. betle* L. was best effective when

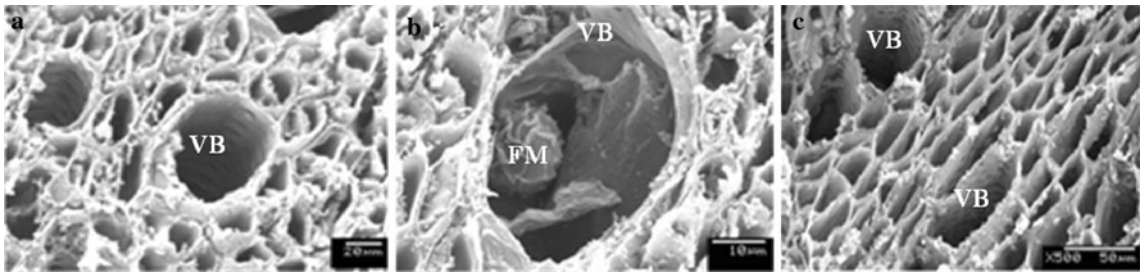

Fig. 4 Scanning electron microscopic slides showing **a** vascular bundles (VB) of tomato plants in healthy control; **b** *Fusarium* infestation (FM) in vascular bundles (VB) of tomato plants in fungal control and **c** clear vascular bundles (VB) showing no *Fusarium* infestation in the plants grown in soil treated with 1% (w/w) chloroform extract of *P. betle* L. (PbC)

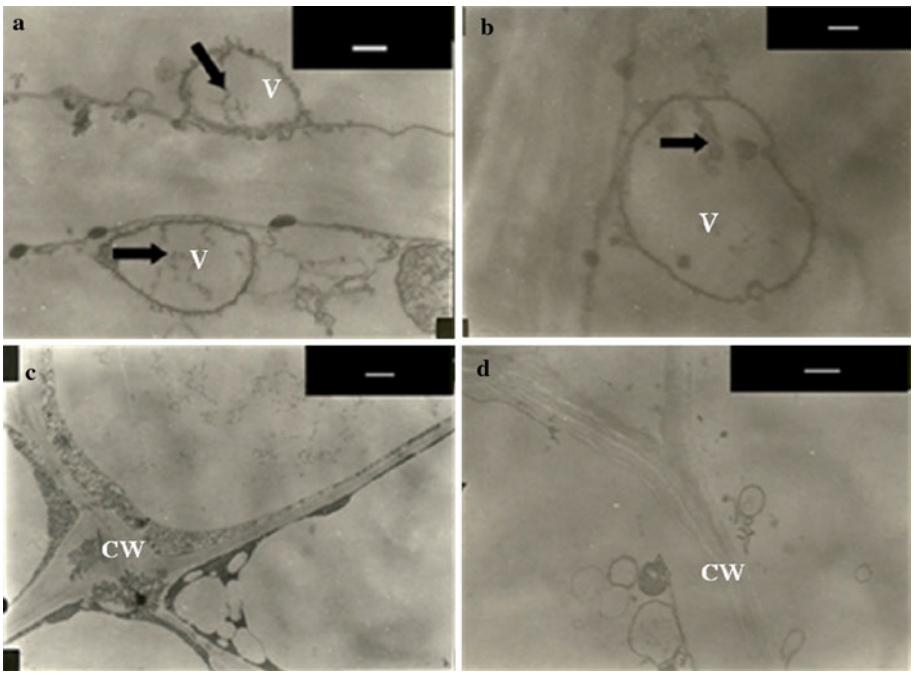

Fig. 5 Accumulation of total phenolics (shown by *arrow*) in vacuoles (V) of **a** healthy root tissue, *Bar* 0.37 µm; **b** *Fusarium-infested* root tissue, *Bar* 0.37 µm; **c** cell wall (CW) in healthy control, *Bar* 1.25 µm and **d** cell wall in *Fusarium-infested* root tissue, *Bar* 1.25 µm

stored at 4°C. Terpenoids were observed to be present in PbC amongst other phytochemicals viz. saponins, phlobatanins, flavonoids, phytosterols, phenols and tannins. Since delayed amendment of PbC in soil after 15 days of *Fusarium* F1 infestation could reduce the disease incidence from 88 to 44%, it can be speculated that some unknown volatile aromatic compounds present in the form of terpenoids from PbC may help in conferring disease resistance inside the vascular tissues of tomato plants. Minerdi et al. (2009) demonstrated that small volatile organic compounds (VOCs) sesquiterpenes, mainly caryophyllene, emitted from the wild type strain negatively influence the mycelial growth of different *formae speciales* of *F. oxysporum*.

High content of phenolics may be an essential component for defense against various pathogens that constantly challenge the plant body. Phenolics from various plant sources and their contribution to antimicrobial and other biological responses are well documented (Benner 1993; Bennet and Wallsgrove 1994). It was observed that there was a decline in the accumulation of total phenolics in the healthy control and the PbC amendments after 21 days of observation. But the *Fusarium*-infested treatments showed more accumulation of total phenolics even after 21 days of treatment, and reached the maximum at 28 days of observation. Decrease in the phenolics may be attributed to strengthening of the plant cell walls by polymerization into lignans and lignins (Randhir and Shetty 2005). Phenolic compounds produced by plants are formed through phenylpropanoid metabolism. Since free phenolics can be cytotoxic in the cytoplasm, plants sequester these compounds in the vacuole or deposit them in the cell wall. Once the phenolic acids reach the cell wall, they may be either ester- or ether- linked to the cell wall polysaccharides or hemicelluloses, or polymerized into lignin (Lewis and Yamamoto 1990). The role of lignins and related polymers has been closely correlated with the defense responses of several plants (Vance et al. 1980). Phenols occur constitutively, whereas others are formed in response to pathogen ingress and associated as part of an active defense response in the host (Nicholson and Hammerschmidt 1992). They are known to confer resistance either directly or indirectly through activation of postinfection responses in the hosts (De Vecchi and Matta 1989). Post-infection activation of phenol metabolism in xylem vessels of tomato has been demonstrated by Matta et al. (1988). The increase in the synthesis and accumulation of total phenolics in the plants grown in *Fusarium*-infested soil may be due to resistance and overcome the stress created by the invading *Fusarium*. It further suggests that only an increase in the synthesis and accumulation of total phenolic compounds in the plant body is insufficient to resist and check the entry of *Fusarium*. So proper sequestration of these phenolic compounds in the form of lignifications in the cell wall is necessary for the plant defense mechanism to confer resistance and overcome the entry of pathogens.

Generally biocontrol and plant secondary metabolites have been known to stimulate the production of phenolics and other compounds that constitutively help the plants in disease resistance. Amendment of PbC in soil reduced the *Fusarium* soil population. The extract effectively checks the *Fusarium* population and its growth in soil and gradually reduces disease symptoms and renders effective growth to tomato plants. However, little is known about the ability of PbC to stimulate higher levels of defense enzymes and host defense related compounds. The characterization of the secondary metabolite from the crude chloroform extract of *Piper betle* L. was determined by other workers in their study (Parmar et al. 1998), but further investigations are required. Thus, isolation and identification of active compounds associated with antifungal activity from chloroform extract of *Piper betle* L. may serve as a promising alternative to chemical fungicides and in management of *Fusarium* wilt of tomato and other soil borne pathogens.

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